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Inhibition of GM-CSF Receptor Function by Stable RNA Interference in a NOD/SCID Mouse Hematopoietic Stem Cell Transplantation Model

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ABSTRACT

RNA interference (RNAi) describes a highly conserved mechanism of sequence-specific posttranscriptional gene silencing triggered by double-stranded RNA (dsRNA). Whereas RNAi is applied to study gene function in different organisms and in variant cell types, little is known about RNAi in human hematopoietic stem and progenitor cells and their myeloid progeny. To address this issue, short hairpin RNAs (shRNA) were designed to target the common β-chain of the human receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 (β-GMR). These receptors regulate proliferation, survival, differentiation, and functional activity of hematopoietic cells. In addition to markedly inhibiting mRNA and protein expression, anti-β-GMR shRNAs were also found to inhibit receptor function in a cell culture model. Furthermore, lentiviral gene transfer of shRNA expression cassettes into primary normal $CD34^+$ cells selectively inhibited colony formation of transduced progenitors when stimulated with GM-CSF/IL-3 but not when stimulated with cytokines that do not signal via β-GMR. Finally, anti-β-GMR shRNAs had no detectable effect on engraftment or lineage composition of lentivirally transduced human $CD34^+$ cells transplanted into NOD/SCID mice. However, the growth defect of transduced colony-forming cells under stimulation with GM-CSF/IL-3 remains unchanged in bone marrow cells harvested from individual NOD/SCID mice 6 weeks after transplantation. These data indicate that lentiviral gene transfer of shRNA expression cassettes may be used to induce long-term RNAi in human hematopoietic stem and progenitor cells for functional genetics and potential therapeutic intervention.

INTRODUCTION

RNA INTERFERENCE (RNAi) DESCRIBES a highly conserved regulatory mechanism of sequence-specific RNA depletion that is initiated by homologous double-stranded RNA (dsRNA). RNAi-mediated gene silencing can be triggered by variant forms of dsRNA, such as small hairpin RNA (shRNA) or small interfering RNA (siRNA), and it is now being used as a tool for functional genomics in multiple organisms (Fire et al., 1998; Ngo et al., 1998; Gonczy et al., 2000; Fraser et al., 2000; Catalanotto et al., 2000; Wianny and Zernicka-Goetz, 2000; Elbashir et al., 2001; Pal-Bhadra et al., 2002; Ashrafi et al., 2003; Kamath et al., 2003; Roignant et al., 2003). In mammalian cells, including human, RNAi induces transient gene silencing after a single siRNA application, whereas stable intracellular transcription of RNAi triggers can induce long-term inhibition of gene expression (Devroe and Silver, 2002; Barton and Medzhitov, 2002; Brummelkamp et al., 2002; Abbas-Terki et al., 2002; Xia et al., 2002; Rubinson et al., 2003; Kunath et al., 2003; Stewart et al., 2003; Scherr et al., 2003a).

Hematopoiesis is a hierarchically ordered system with a pool of self-renewing and pluripotent hematopoietic stem cells (HSC) generating committed progenitors that finally differentiate into mature blood cells of all hematopoietic

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lineages. The proliferation and differentiation of hematopoietic cells are partly regulated by hematopoietic cytokines, such as interleukin-3 (IL-3), erythropoietin (EPO), stem cell factor (SCF), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte CSF (G-CSF) (for review, see Metcalf, 1989). Hematopoietic cytokines initiate their effects by binding to specific cell surface receptors. On ligand binding, these receptors become activated and subsequently transducer specific intracellular signals involving induction of tyrosine kinase activity (for review, see Ihle, 1995).

Until now, RNAi in hematopoiesis has been studied mostly in lymphatic cell lines or primary T lymphocytes in the context of HIV infection (Martinez et al., 2002; McManus et al., 2002; Capodici et al., 2002; Coburn and Cullen, 2002; Jacque et al., 2002; Banerjea et al., 2003; Qin et al., 2003). In contrast, little is known about RNAi in $CD34^+$ stem and progenitor cells and their myeloid progeny. Our group recently reported first results on specific RNAi against the common β-chain of the receptors for GM-CSF, IL-3, and IL-5 (referred to as β-GMR) in primary $CD34^+$ colony-forming cells. The human receptors for GM-CSF, IL-3, and IL-5 have unique α-chains required for ligand binding but share a common β-chain (β-GMR) essential for intracellular signal transduction. Using the β-GMR model that allows specific analysis of receptor function by the selective choice of cytokine stimulation for *in vitro* cultures, we demonstrated stable RNAi in $CD34^+$ colony-forming progenitor cells on lentiviral gene transfer of shRNA expression cassettes (Scherr et al., 2003a). In contrast to colony-forming cells, which represent late hematopoietic progenitors, there are no reports to date on stable RNAi in human HSC. For example, it is not known to what extent primary human HSC can be genetically modified to inhibit endogenous gene expression by stable RNAi. Additionally, the impact of expression of sh/siRNA on engraftment capability and differentiation potential of HSC has not been reported.

To address these questions, we expressed selected anti β-GMR shRNAs from lentiviral H1 expression cassettes, and we demonstrate the inhibition of mRNA and protein expression as well as RNAi-mediated inhibition of GMR signal transduction in a cell culture model. We performed xenotransplantation of primary $CD34^+$ cells into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and used NOD/SCID repopulating cells (SRC) as a surrogate to analyze human HSC (Dick et al., 1997). We show specific and nearly identical inhibition of β-GMR function in colony-forming cells both before and after transplantation of $CD34^+$ cells into NOD/SCID mice without detectable effects of multilineage engraftment of SRCs. These data demonstrate that stable RNAi can be induced in human HSC by lentiviral gene transfer and can mediate long-term and functional gene silencing of an endogenous hematopoiesis-specific gene.

MATERIALS AND METHODS

shRNA synthesis and construction of H1-shRNA expression cassettes

Five different DNA oligonucleotides corresponding to positions 1466–1484, 1495–1513, 1775–1793, 2095–2113, and 2194–2212 of the sequence of the common β-chain of the human receptors for GM-CSF, IL-3, and IL-5 were chemically synthesized, including overhang sequences from a 5′ BglII and a 3′ SalI restriction site for cloning purposes (BioSpring, Frankfurt, Germany). The numbering of β-GMR nucleotides refers to Hayashida et al. (1990) (GeneBank accession No. M59941). The oligonucleotide sequences were as follows: FP1466, 5′-GATCCCCCCCCCAGCAAGAGCCAC*CTG*TTCAAGAGA*CAGGTGGCTCTTGCTGGGG*-TTTTTTGGAAG-3′; RP1466, 5′-TCGACTTCCAAA-AAA*CCCCAGCAAGAGCCACCTG*TCTCTTGAA-*CAGGTGGCTCT*TGCTGGGGGGG-3′; FP1495, 5′-GA-TCCCCC*GGGAGCGCAGAGCTTTGG*TTCAAGAGA*CCAAAGCTCTGCGCTCCCG*TTTTTTGGAAG-3′; RP1495, 5′-TCGACTTCCAAAAAA*CGGGAGCGCAG-AGCTTTGG*TCTCTTGAA*CCAAAGCTCTGCGCTCC-CG*GGG-3′; FP1775, 5′-GATCCCCC*AGGCTTCCAG-CTTTGAC*TTCAAGAGA*AGTCAAAGCTGGAAGCCT G*TTTTTTGGAAG-3′; RP1775, 5′-TCGACTTCCAA-AAAA*CAGGCTTCCAGCTTTGACT*TCTCTTGAA*AGT CAAAGCTGGAAGCCTG*GGG-3′; FP2095β, 5′-GATC-CCC*GGACAGCCCTGTGGCTATA*TTCAAGAGA*TA-TAGCCACAGGGCTGTCC*TTTTTTGGAAG-3′; RP2095β, 5′-TCGACTTCCAAAAAA*GGACAGCCCT-GTGGCTATA*TCTCTTGAA*TATAGCCACAGGGCT-GTCC*GGG-3′; FP2194, 5′-GATCCCCC*TCAGGGGC-CTCGTCTGTC*TTTCAAGAGA*GACAGACGAGGCCC CTGAG*TTTTTTGGAAG-3′; RP2194, 5′-TCGACTTC-CAAAAAA*CTCAGGGGCCTCGTCTGTC*TCTCTT-GAA*GACAGACGAGGCCCCTGAG*GGG-3′. The non-complementary 9-nt loop sequences are underlined, and each sense oligonucleotide harbors a stretch of T as a polymerase III (pol III) transcription termination signal. Corresponding oligonucleotides were annealed and inserted 3′ of the H1-RNA promoter into the BglII/SalI-digested pBS-H1 plasmid to generate, e.g., pBS-H1-2095β (Scherr et al., 2003a). The correct sequence and insertion were confirmed by DNA sequencing for each plasmid. The controls pH1-GL4, pH1-121GFP, and pH1-243GFP were described earlier (Scherr et al., 2003a).

Construction of lentiviral vectors

pHR′-SIN-SEW (Demaison et al., 2002) was used to generate lentiviral transgenic plasmids containing H1-siRNA expression cassettes located in the U3 region of the Δ3′-LTR using an SnaBI-restriction site introduced by site-directed mutagenesis (Fig. 1B) (Scherr et al., 2003a). The location of the H1 expression cassette in the

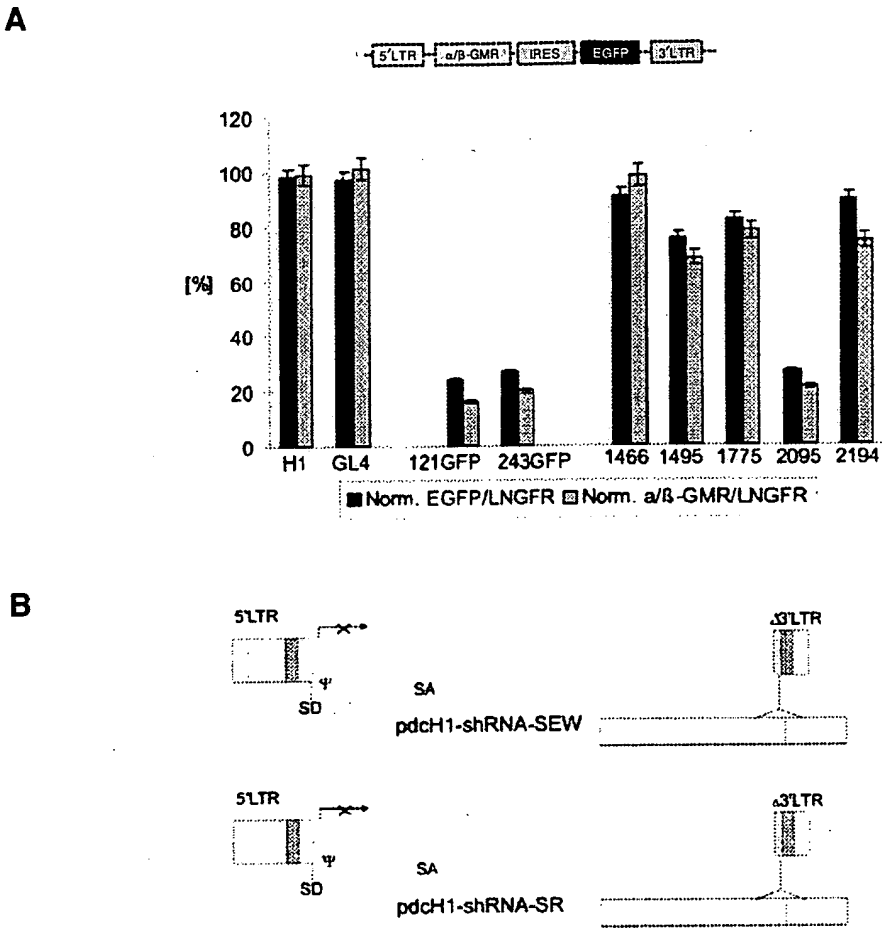

FIG. 1. siRNAs expressed from lentiviral vectors. (**A**) Flow cytometry analysis of α/β-GMR surface expression (stippled) and EGFP fluorescence (black) in BHK-21 cells cotransfected with a bicistronic expression plasmid encoding α/β-GMR and EGFP and individual shRNAs. LNGFR expression was used for normalization. H1, GL4, control shRNAs; 121GFP, 243GFP, anti-GFP shRNAs; 1466, 1495, 1775, 2095, 2194, anti-β-GMR shRNAs. (**B**) Schematic representation of the lentiviral transgene plasmids encoding shRNAs. The shRNA is transcribed from a human H1-RNA promoter inserted into the U3 region of the Δ3′-LTR of the lentiviral vector to obtain a double-copy vector. The vector encodes either EGFP or RFP as a marker gene driven by the spleen focus forming virus promoter (SSFV-LTR) and harbors a cPPT/CTS sequence. The WPRE element for enhanced transgene expression was only inserted into the pdcH1-shRNA-SEW transgene plasmid. 5′-LTR HIV-1 5′-LTR; Δ3′-LTR, HIV-1 self-inactivating (SIN) 3′-LTR, GA deleted *gag* sequence; RRE, rev-responsive element; SD, splice donor site; SA splice acceptor site; Ψ, packaging signal.

Δ3′-LTR results in duplication during reverse transcription, as described earlier (Scherr et al., 2003a). To generate double-copy (dc) H1-siRNA transgenic plasmids, the pH1-2095β and pH1-GL4 plasmids were digested with SmaI and HincII, and the resulting DNA fragments (360 nt) were blunt-end ligated into the SnaBI site of pHR′-SIN-SEW-SnaBI to generate pdcH1-2095β-SEW and pdcH1-GL4-SEW, as well as in the pHR′-SIN-SR-SnaBI to generate pdcH1-2095β-SR and pdcH1-GL4-SR, respectively (Scherr et al., 2003a). The SEW and SR lentiviral constructs encode enhanced green fluorescence protein (EGFP) and RFP as reporter genes, respectively.

Cell culture

The adherent cell lines BHK-21, 293T, and SC-1 were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 2 mM L-glutamine (GIBCO, Grand Island, NY). The cloning and functional characterization of α/β-GMR have been described earlier in detail (Eder et al., 1994; Kafert et al., 1999b). BaF3/α/β-GMR clones and BaF3/α/β-GMR-IRES-EGFP clones were generated as described, and individual clones were isolated by limiting dilution. The cells were grown in RPMI 1640 supplemented with 10% FBS and MuIL-3 (supplied as 10% conditioned medium from Wehi3B cells) or HuGM-CSF (20 ng/ml or as indicated).

Proliferation assay

GM-CSF-dependent proliferation was analyzed by trypan blue exclusion assay. Briefly, 5×10^4 BaF3/α/β-GMR cells/ml were grown in 96-well plates in a total volume of 200 μl with increasing amounts of HuGM-

CSF (0, 0.1, 0.5, 1, and 5 ng/ml). Cell numbers were analyzed after 72 hours by trypan blue exclusion.

Transfection

Transfections of BHK-21 cells were performed using the cationic lipid Lipofectamine 2000 (Life Technologies, Gaithersburg, MD). Cells (1×10^5) were seeded in a 24-well plate and transfected after 8 hours. The cotransfection was performed with three different plasmids: 1 μg MSCV-α/βGMR-IRES-GFP, 0.1 μg LNSN plasmid, encoding the low-affinity nerve growth factor receptor, and 1 μg shRNA expression vector, formulated into liposomes and added to cells as described by the manufacturer. After 48 hours, EGFP and low-affinity nerve growth factor receptor (LNGFR) expression was assessed by FACS and fluorescence microscopy (Axiovert 300, Nikon, Düsseldorf, Germany). To monitor LNGFR expression, 1×10^5 cells were incubated with phycoerythrin (PE) antihuman LNGFR antibody (mouse IgG) (PharMingen, San Diego, CA) for 30 minutes at 4°C, diluted with 1× phosphate-buffered saline (PBS), and collected by centrifugation for 10 minutes at 2000 rpm. To analyze α/β-GMR expression, 1×10^5 cells were incubated with a monoclonal antibody (mAb) directed against the extracellular domain of α-GMR (GM-CSFα S-20, dilution 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at 4°C, followed by incubation with an allophycocyamin (APC)-labeled goat anti-mouse antibody (Dianova, Hamburg, Germany).

Preparation of recombinant lentiviral supernatants and lentiviral transduction

VSV.G-pseudotyped lentiviral particles were generated by calcium phosphate cotransfection of 293T cells, and viral supernatants were concentrated as previously described (Scherr et al., 2002). dcH1-shRNA-SEW and dcH1-shRNA-SR lentiviral preparations were titered in triplicate by serial dilutions of the concentrated vector stocks on 1×10^5 SC-1 cells in 24-well plates. The number of EGFP-positive or RFP-positive cells was analyzed 72 hours posttransduction by FACS analysis (FACS-Calibur, Becton Dickinson, Mountain View, CA), typically yielding titers of about $1–5 \times 10^8$ IU/ml.

Lentiviral supernatants were used to transduce BaF3/α/β-GMR cells. Lentiviral transduction, including spin occulation, was performed as described earlier (Scherr et al., 2002). Two days after transduction, cells were expanded as bulk populations and analyzed by RT-PCR or FACS.

Real-time RT-PCR

Cytoplasmatic RNA was isolated using the RNeasy mini-spin columns (Qiagen, Hilden, Germany) from 1×10^6 BaF3/α/β-GMR cells and digested with 2 U DNase I (Roche, Mannheim, Germany) for 1 hour at 37°C, followed by phenol chloroform extraction and ethanol precipitation. RNA was reverse transcribed into cDNA in a total volume of 20 μl using MMLV-reverse transcriptase (Invitrogen, San Diego, CA) and random hexamer primers under standard conditions. Real-time Taqman RT-PCR of α/β-GMR and murine GAPDH was performed as described previously (Scherr et al., 2003b; Kafert et al., 1999a).

Northern blot analysis

Total RNA from transduced BaF3/α/β-GMR-IRES-GFP cells was isolated using Trizol according to the manufacturer's instruction (Invitrogen). RNA (20 μg) was subjected to 16% denaturing polyacrylamide gel electrophoresis and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by electroblotting. The hybridization and washing steps were carried out at 37°C. Membranes were probed with either ^{32}P-labeled 19-nt GFP or α/β-GMR oligonucleotide corresponding to the sense strand of the respective 121 and α/β-GMR siRNAs and were subsequently visualized by autoradiography. Endogenous U6 snRNA served both as an internal size standard and as a loading control (U6snRNA probe: 5′-TATGGAACGCTTCACGAATTTGC-3′).

Isolation and lentiviral transduction of peripheral blood-derived CD34$^+$ cells

G-CSF-primed CD34$^+$ cells were harvested by leukapheresis from four healthy volunteers, purified to ≥98% CD34$^+$ content by magnetic cell sorting (Clini MACS, Miltenyi Biotech, Bergisch Gladbach, Germany), and cryopreserved in liquid nitrogen. Lentiviral transduction of CD34$^+$ cells was performed twice as described previously (Scherr et al., 2002). Methylcellulose colony assays with SCF (20 ng/ml) + G-CSF (10 ng/ml) + TPO (10 U/ml) or GM-CSF (20 ng/ml) + IL-3 (10 ng/ml) were performed as described (Schiedlmeier et al., 2000). The cytokines were all from R&D Systems (Abingdon, U.K.).

Transplantation of CD34$^+$ cells into NOD/SCID mice

NOD/SCID mice were kept and transplanted with transduced and mock-transduced human CD34$^+$ cells (2×10^6 cells in 300 μl IMDM per mouse) and irradiated fibroblasts producing human IL-3 (Maingen, Frankfurt, Germany). Mice were killed 6 weeks after transplantation, and engraftment and multilineage RFP expression were analyzed by FACS (Schiedlmeier et al., 2000).

RESULTS

Selection of anti-β-GMR shRNAs

Several anti-β-GMR shRNAs were synthesized targeting 19 nt of the β-GMR sequence starting at β-GMR po-

sition 1466, 1495, 1775, 2095, and 2194, respectively, and were subsequently cloned into H1-shRNA expression cassettes. To quantify shRNA-triggered gene silencing, BHK-21 cells were cotransfected with (1) a bicistronic expression plasmid encoding a chimeric α/β-GMR identical to β-GMR 3′ of nucleotide 1424 (Eder et al., 1994) and EGFP (Fig. 1A), (2) an expression plasmid with the LNGFR cDNA used for normalization, and (3) individual shRNAs, including two anti-EGFP shRNAs as reported earlier (Scherr et al., 2003a). The chimeric α/β-GMR can be activated by human GM-CSF and mediates proliferation and survival in transfected cells, thus allowing study of GMR function on expression of a single transgene, as opposed to the wild-type α-GMR + β-GMR (Eder et al., 1994; Kafert et al., 1999b). As shown in Figure 1A, expression of both EGFP fluorescence and α/β-GMR was measured by FACS analysis 24 hours after cotransfection with each shRNA construct and normalized for LNGFR expression. The effects of individual shRNAs on both EGFP fluorescence and α/β-GMR surface expression were very similar in all cases. However, only one of five anti-β-GMR shRNAs (2095) inhibited target gene expression to an extent similar to both anti-EGFP shRNAs (up to 80%). In contrast, the remaining four β-GMR shRNAs induced only low or marginal inhibition of EGFP fluorescence and α/β-GMR surface expression.

The H1-2095 shRNA cassette was subsequently in-

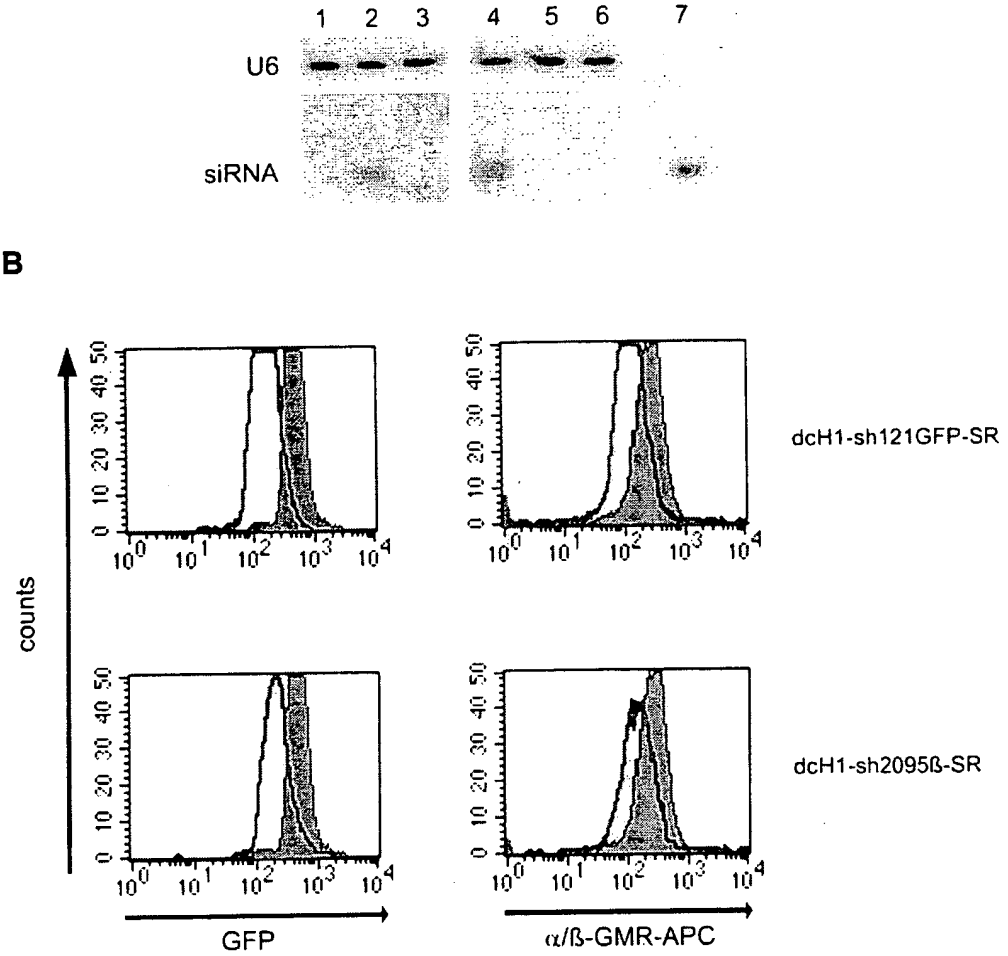

FIG. 2. Lentivirus-mediated gene silencing in BaF3-α/β-GMR-IRES-GFP cells. (**A**) Northern blot analysis depicts cellular siRNA expression after transduction of BaF3-α/β-GMR-IRES-GFP cells with dcH1-2095β-SR (lanes 2 and 5), dcH1-121GFP-SR (lanes 1 and 4), and dcH1-GL4-SR (lanes 3 and 6). Lane 7, a 21-nt long chemically synthesized 121 GFP siRNA duplex. The blot on the left (lanes 1–3) was probed with ^{32}P-labeled 19-nt β-GMR oligonucleotide corresponding to the sense strand of the 2095β siRNA, and the blots in the middle and on the right (lanes 4–7) were hybridized with ^{32}P-labeled 19-nt GFP oligonucleotide corresponding to the 121GFP siRNA. A shorter exposure to visualize U6 snRNA as loading control is shown in the upper panel. (**B**) Surface expression of α/β-GMR (right) and EGFP fluorescence (left) was measured at day 6 after a single transduction with either dcH1-121GFP-SR (**top**) or with dcH1-2095β-SR (**bottom**). The filled curves show untransduced BaF3-α/β-GMR-IRES-GFP cells as control.

serted into two lentiviral transgene plasmids to generate pdcH1-2095-SR and pdcH1-2095-SEW, respectively (Fig. 1B). Both constructs are optimized for lentiviral transduction by the cPPT/CTS sequence and harbor the H1-shRNA cassette located in the Δ3′-LTR, leading to their duplication during reverse transcription (dc constructs). pdcH1-2095-SR and pdcH1-2095-SEW differ in the reporter gene encoded (RFP and EGFP), and pdcH1-2095-SEW but not pdcH1-2095-SR contains a WPRE (W) element to enhance EGFP expression. Lentiviral supernatants were generated, and expression of siRNAs after lentiviral gene transfer was demonstrated by Northern blotting of RNA isolated from BaF3 cells expressing α/β-GMR and EGFP from the bicistronic transcript

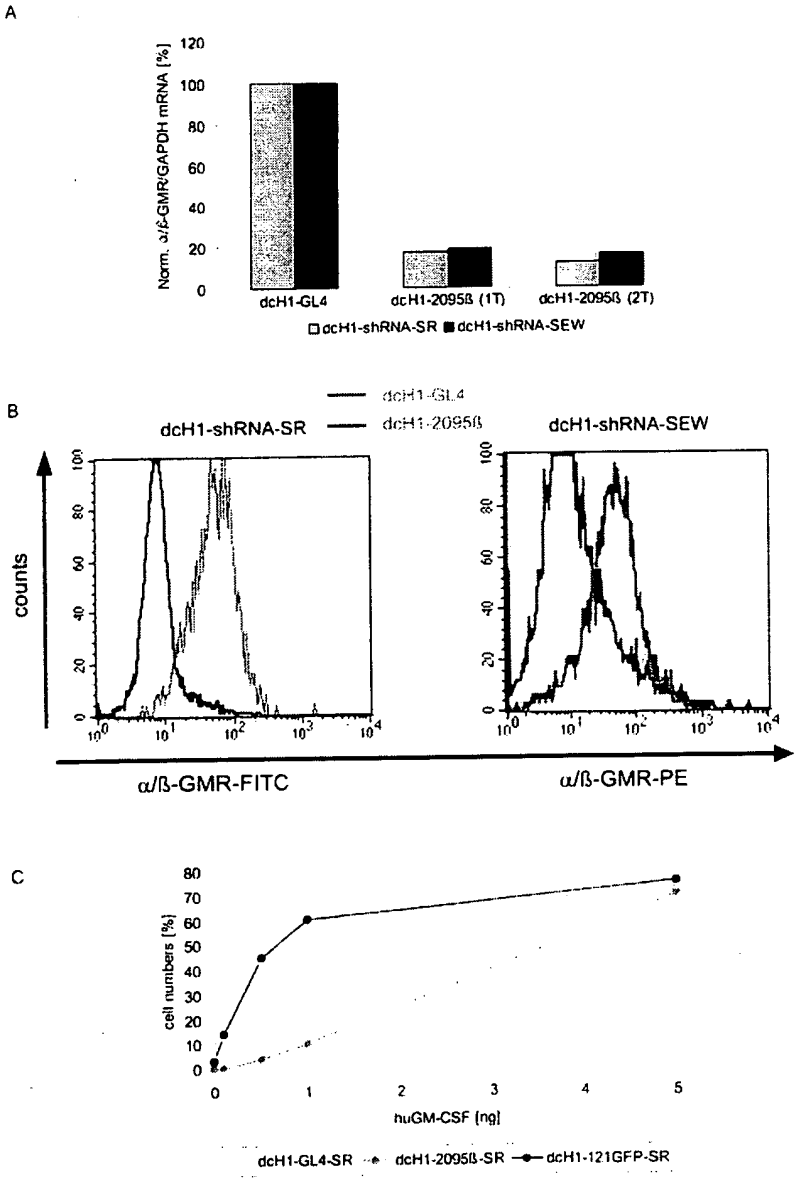

FIG. 3. Lentivirus-mediated gene silencing in BaF3-α/β-GMR cells (**A**) α/β-GMR mRNA levels were measured by real-time RT-PCR 6 days after repeated lentiviral transduction (1T, single transduction; 2T, double transduction) and were normalized in comparison to GAPDH expression. α/β-GMR expression after transduction with control shRNA dcH1-GL4 was set to 100%. dcH1-GL4-SEW and dcH1-GL4-SR encode control shRNA, and dcH1-2095β-SEW and dcH1-2095β-SR encode anti-β-GMR shRNAs. (**B**) Surface expression of α/β-GMR was measured at day 6 after the second transduction with either dcH1-shRNA-SR (**left**) or dcH1-shRNA-SEW (**right**) viruses. BaF3-α/β-GMR cells transduced with dcH1-GL4-SR (**left, panel, right curve**) or dcH1-GL4-SEW (**right panel, right curve**) served as controls. (**C**) Inhibition of GM-CSF-mediated cell proliferation in a dose-dependent manner (ranging from 0 to 5 ng/ml HuGM-CSF) by transduction with dcH1-2095-SR but not with control dcH1-GL4-SR or dcH1-121GFP-SR. Cell numbers of cultures stimulated with MuIL-3 were set 100%.

schematically shown in Figure 1A. As shown in Figure 2A, anti-β-GMR (lane 2) and anti-121GFP (lane 4) siRNAs can be generated from proviral dcH1-2095-SR and dcH1-121GFP-SR, respectively. In addition, equivalent inhibition of α/β-GMR surface expression and EGFP fluorescence mediated by either anti β-GMR or anti-121GFP siRNAs was observed in transduced BaF3α/β-GMR-IRES-EGFP cells (Fig. 2B).

Function of anti-β-GMR sh/siRNAs in a reporter cell line

To evaluate the efficiency of siRNA 2095β, BaF3 cells expressing α/β-GMR (BaF3/α/β-GMR) were transduced with either dcH1-2095-SR or dcH1-2095-SEW lentiviral supernatants. α/β-GMR mRNA levels were measured using real-time RT-PCR after one or two rounds of lentiviral transduction. As shown in Figure 3A, both lentiviral preparations inhibited target mRNA expression by about 80% compared with control shRNA. In addition, this decrease in target mRNA levels was not significantly enhanced by a second lentiviral transduction. FACS analysis of α/β-GMR protein expression on the cell surface revealed a marked and slightly higher reduction on transduction with dcH1-2095-SR compared with dcH1-2095-SEW (Fig. 3B). Interestingly, >95% of BaF3/α/β-GMR cells exhibited green and red fluorescence, respectively, indicating a 95% or higher transduction rate (data not shown). Based on these results, all further experiments were performed with dcH1-2095-SR lentiviral preparations.

To analyze RNAi-mediated functional defects in α/β-GMR signaling, BaF3/α/β-GMR cells were cultured in medium containing different concentrations of HuGM-CSF. BaF3 cells are murine pro-B cells that require MuIL-3 for survival and proliferation. However, expression of α/β-GMR enables culture in either MuIL-3 or HuGM-CSF (Eder et al., 1994). As shown in Figure 3C, transduction with dcH1-2095-SR, but not with control dcH1-GL4-SR or dcH1-121GFP-SR, inhibited GM-CSF-mediated cell proliferation in a dose-dependent manner. As BaF3/α/β-GMR cells express α/β-GMR from a monocistronic transcript that does not contain EGFP, anti-GFP siRNA had no effect on α/β-GMR gene expression in these cells.

Function of anti-β-GMR sh/siRNAs in primary CD34⁺ SRC and progenitor cells

To analyze RNAi in HSC and their progeny, lentivirally transduced CD34⁺ cells were transplanted into NOD/SCID mice. The multilineage engraftment of lentivirally transduced human CD34⁺ cells harvested 6

TABLE 1. LYMPHOHEMATOPOIETIC RECONSTITUTION OF NOD/SCID MICE[a]

	Mouse 1 dcH1-GL4-SR	*Mouse 2 dcH1-GL4-SR*	*Mouse 3 dcH1-2095β-SR*	*Mouse 4 dcH1-2095β-SR*	*Mouse 5 dcH1-2095β-SR*
HuCD45⁺	87	58	75	94	60
RFP⁺/HuCD45⁺	8	8	10	8	7
CD34⁺/HuCD45⁺	3	3	3	4	3
RFP⁺/CD34⁺	NE[b]	NE	NE	NE	NE
CD33⁺/HuCD45⁺	65	70	70	69	66
RFP⁺/CD33⁺	7	9	13	7	11
CD38⁺/HuCD45⁺	61	55	58	63	62
RFP⁺/CD38⁺	11	13	14	9	10
CD19⁺/HuCD45⁺	32	28	28	27	33
RFP⁺/CD19⁺	6	6	5	3	3
CD14⁺/HuCD45⁺	6	9	8	5	9
RFP⁺/CD14⁺	NE	NE	NE	NE	NE

[a]The ratio of human cells (HuCD45⁺), lymphohematopoietic subpopulations (CD34⁺, CD33⁺, CD38⁺, CD19⁺, and CD14), and the level of RFP expression are given in percent. RFP⁺/CD45⁺ represents the percentage of RFP⁺ cells within the human CD45⁺ cell fraction.

[b]NE, not evaluable; subpopulations with <10% engraftment were considered not suitable for accurate analysis of lentiviral transduction.

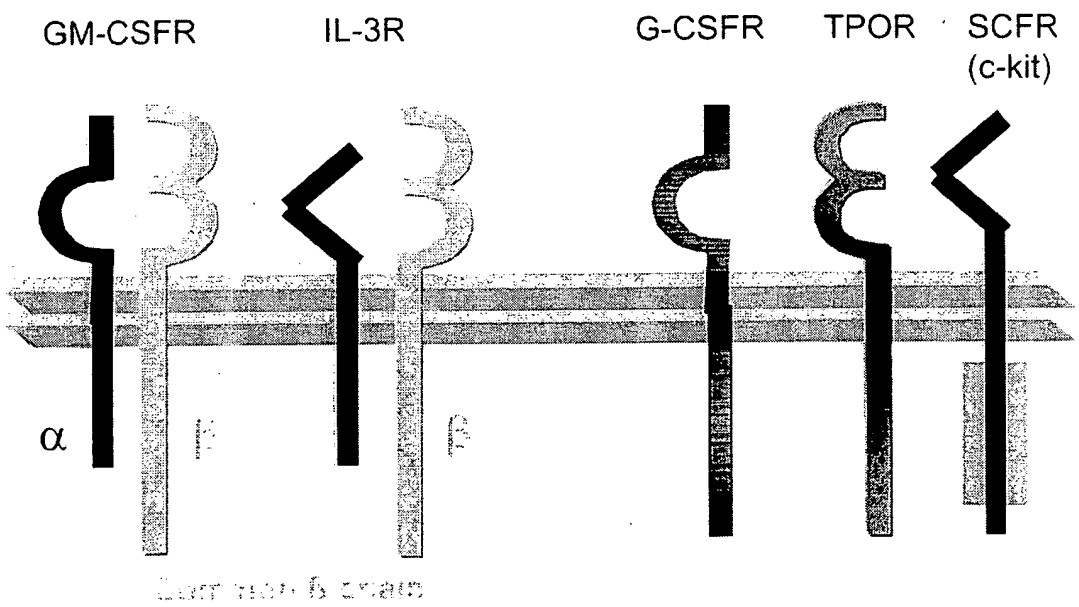

FIG. 4. Hematopoietic cytokine receptors. Illustrated here are the human GM-CSF and IL-3 receptors with the common β-chain at left and the receptors for G-CSF, TPO, and SCF at right. The shaded box in SCFR (c-*kit*) indicates its cytoplasmic tyrosine kinase domain.

weeks after transplantation is shown in Table 1. Analysis of human cells in murine bone marrow revealed no difference between mice engrafted with dcH1-2095-SR or dcH1-GL4-SR transduced CD34$^+$ cells with 58%–94% of all nucleated bone marrow cells of human origin. In addition, the ratio of transduced, RFP+ cells in the myeloid and B lymphoid compartment was as high as 10% in human CD45$^+$ cells and was similar in control and anti-β-GMR siRNA mice.

To study functional gene silencing, clonogenic assays of hematopoietic progenitor cells were performed before and after transplantation of CD34$^+$ cells into NOD/SCID mice. This *in vitro* culture allows functional analysis of specific cytokine receptors depending on the cytokines used for stimulation. As schematically shown in Figure 4, stimulation with GM-CSF and IL-3 requires functional β-GMR, whereas signal transduction initiated by G-CSF, SCF, and TPO is independent of β-GMR. Accordingly, normal CD34$^+$ cells transduced with control dcH1-GL4-SR lentivirus generate RFP$^+$ and RFP$^-$ colonies at a nearly identical ratio under stimulation with either GM-CSF/IL-3 or G-CSF/SCF/TPO, with transduction rates between 5% and 18% in the G-CSF/SCF/TPO condition, which is not affected by RNAi against β-GMR (Table 2A). In contrast, the ratio of RFP$^+$ colonies derived from CD34$^+$ cells transduced with dcH1-2095-SR decreased by an average of about 60% when cultured in the presence of GM-CSF and IL-3, compared with G-CSF/SCF/TPO. The transduction rates after infection with dcH1-2095-SR were higher than those observed with dcH1-GL4-SR and ranged between 11% and 52% for individual donors (determined under stimulation with G-CSF/SCF/TPO).

Identical clonogenic cultures were performed with cells harvested from bone marrow of individual NOD/SCID mice engrafted with either dcH1-2095-SR or dcH1-GL4-SR-transduced CD34$^+$ cells. Again, the ratio of RFP$^+$ colonies was similar for both cytokine conditions in the dcH1-GL4-SR control mice (Table 2B). In contrast, the ratio of RFP-transduced colonies decreased by an average of 62% under stimulation with GM-CSF/IL-3 compared with G-CSF/SCF/TPO in the three mice engrafted with dcH1-2095-SR-transduced CD34$^+$ cells.

DISCUSSION

We analyzed GM-CSF receptor function to study RNAi in primary human CD34$^+$ hematopoietic stem and progenitor cells. We selected anti-β-GMR shRNAs based on their capacity to reduce target mRNA levels in a transient cotransfection assay. This approach uses bicistronic transcripts and allows rapid and quantitative detection of RNAi by FACS analysis of EGFP fluorescence. In our experiments, RNAi induced an equivalent reduction of both mRNAs of the bicistronic transcript independent of the 5′ or 3′ location of the respective target gene for RNAi. Only one of five anti-β-GMR shRNA, but both pretested anti-GFP shRNAs (Scherr et al., 2003a) induced effective gene silencing in this assay.

Lentiviral gene transfer was used to stably introduce H1-shRNA expression cassettes into the target cell genome. Constitutive transcription of shRNAs can induce long-term gene silencing, as demonstrated in earlier studies (Scherr et al., 2003a). The anti-β-GMR shRNA was

TABLE 2A. COLONY FORMATION OF CD34$^+$ HEMATOPOIETIC PROGENITOR CELLS[a]

	CD34$^+$ sample	*Number RFP$^+$ colonies/total colony number(%)*	
		GM-CSF/IL-3	*G-CSF/SCF/TPO*
dcH1-GL4-SR	A	11	10
dcH1-2095β-SR		3	11
dcH1-GL4-SR	B.1	16	18
dcH1-2095β-SR		32	52
dcH1-GL4-SR	B.2	15	17
dcH1-2095β-SR		6	32
dcH1-GL4-SR	C	4	5
dcH1-2095β-SR		7	24
dcH1-GL4-SR	D	19	18
dcH1-2095β-SR		19	32

[a]CD34$^+$ samples enumerated in alphabetical order. Sample B was transduced with different lentiviral vector preparations.

TABLE 2B. COLONY FORMATION OF SRC-DERIVED HEMATOPOIETIC PROGENITOR CELLS

Transplanted mice	*Sample*	*Number RFP$^+$ colonies/Total colony number*	
		GM-CSF/IL-3	*G-CSF/SCF/TPO*
Mouse 1	dcH1-GL4-SR	10	8
Mouse 2	dcH1-GL4-SR	7	9
Mouse 3	dcH1-2095β-SR	9	15
Mouse 4	dcH1-2095β-SR	4	14
Mouse 5	dcH1-2095β-SR	3	11

tested in a murine cell line that expresses a chimeric single chain α/β-GMR, which encodes the entire intracellular domain of β-GMR. A significant RNAi-mediated reduction in target mRNA and surface protein expression was found in BaF3/α/β-GMR cells. In addition, RNAi inhibited α/β-GMR function depending on the GM-CSF dose. Interestingly, these results correspond very well to the inhibition of α/β-GMR function observed on coexpression of a dominant negative splice variant of β-GMR in BaF3/α/β-GMR cells (Wagner et al., 2001), indicating that higher doses of ligand can compensate for lower numbers of functional receptor molecules in this specific model.

We next examined the engraftment and differentiation capacity of human primary CD34$^+$ cells after lentiviral transduction with anti-β-GMR shRNA expression cassettes in the NOD/SCID mouse model. This model is considered a standard tool to analyse SRC as a surrogate for human HSC (Dick et al., 1997). Our data indicate that lentivirus-mediated RNAi targeting the endogenous β-GMR gene did not significantly affect engraftment and lineage composition of hematopoietic cells compared with controls. This is not due to loss of RNAi or transient gene silencing, as the phenotype of β-GMR inhibition remains almost identically conserved in colony-forming cells before and after transplantation, demonstrating long-term functional gene silencing (Tables 2A and 2B). These data may indicate that β-GMR function is not required for engraftment of SRC, as suggested by the fact that many groups omit *in vivo* treatment with HuIL-3 without negative effects on SRC engraftment. Alternatively, transduced cells that express sufficient β-GMR in spite of RNAi may be selected under the treatment conditions used in this study. A higher number of NOD/SCID mice have to be analyzed using different treatment modalities to definitively characterize potential functions of β-GMR in the NOD/SCID xenotransplantation model. Furthermore, we demonstrate that RFP can be used as a reporter gene for lentiviral transduction in the NOD/SCID model. Future studies are necessary to compare the effects of different reporter genes, such as RFP or EGFP, on SRC or hematopoietic progenitor cells.

In summary, our data demonstrate for the first time that

lentiviral transfer of suitable shRNA expression cassettes can induce long-term RNAi in primary SRC without detectable effects on engraftment capability and lineage composition of SRC-derived hematopoietic cells. This approach will allow functional genomics in primary human HSC and their myeloid progeny as demonstrated here for β-GMR. Finally, stable RNAi may eventually be applied as a therapeutic strategy in hematologic diseases characterized by aberrant gene expression either due to mutations as in cancer or derived from foreign infectious organisms.

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